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(54) Title: HEPARIN DERIVATIVES

(57) Abstract

Low molecular weight heparin derivatives and salts thereof are prepared by treating a quaternary ammonium salt of a heparin, dissolved in a heterocyclic organic solvent or in an aprotic solvent, with an alkylating agent having from 6 to 30 carbon atoms at a temperature of about 20° to 60 °C for a prolonged period of time, treating the resultant reaction product at a temperature of about 5° to 120 °C with an inorganic or organic base, such as an alkaline hydroxide, in aqueous solution, and isolating the obtained heparin derivatives in their free form or as alkaline salts thereof. The heparin derivatives obtained according to the procedure of the invention constitute a mixture of mostly depolymerized products with a narrow range of molecular weight. These products are useful as anti-thrombotic agents.

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Heparin derivatives

Background and Summary of the Invention

The present invention concerns new heparin derivatives with pharmacological properties which are modified with respect to those of the heparin preparations currently available on the market and used in anticoagulant therapies. The relationship between the anti-thrombin activity and the activity on the Xa factor and the platelet factor 4 (PF_4) of the new derivatives differs from that of heparin. In other words, their considerable affinity for PF_4 is accompanied by reduced activity on the reaction between anti-thrombin III and thrombin, catalyzed by heparin.

In parallel, the reaction between antithrombin III and the Xa factor, catalyzed by heparin itself, is even more sensitive to the new derivatives. Because of their different ratio of activity from that of heparin, on PF_4 and on the reactions activated by thrombin and Xa factor, the derivatives of the invention are able to prevent thrombosis without the risk of hemorrhage which accompanies the anti-thrombotic action of heparin. It must be noted that hemorrhagic activity can be caused by an excessive action of thrombin inhibition. The ability of the new derivatives of the invention to bind PF_4, to catalyze at low concentrations the reaction activated by the Xa factor, and only at higher concentrations reactions catalyzed by thrombin, make these compounds suitable as drugs for the treatment of arterial and venous thrombosis.

The new heparin derivatives and their salts are obtained by

(1) prolonged treatment of an alkylating (esterifying) agent derived from a hydrocarbon with more than 6 carbon atoms, at room temperature or slightly over, with a quaternary ammonium salt of heparin, dissolved in a heterocyclic organic solvent chosen from N-hydrocarbyl-pyrrolidine-2-one or

N-hydrocarbyl-piperidine-2-one, not substituted or substituted by lower alkyl groups, and their derivatives interrupted in the heterocyclic ring by a heteroatom or hetero group chosen from -O-, -S- or -NH- or in a concentrated solution of one such compound in an aprotic solvent,

(2) treating the reaction product at a high temperature with an organic or inorganic base in aqueous solution,

(3) isolating the heparin derivatives obtained in free form or their alkaline salts, and, if desired,

(4) converting the resulting compounds into the free form or into the alkaline salts or as salts of other metals or organic bases.

The new heparin derivatives, whose aforesaid pharmacological properties are different from those of heparin, also have a modified chemical structure and different molecular weight; their chemical structure has not yet been completely defined. It has however been ascertained that they:

a) constitute a mixture of mostly depolymerized products and therefore have a medium-to-low molecular weight compared the starting products, which are rich in fragments similar to one another and with molecular weights coming within the same narrow range, and with a minimum of fragments which deviate from this mean.

b) Compared to their starting products, they have a notably diminished ratio between the iduronic-type saccharide unit and the glucuronic-type unit.

Other modifications of the chemical structure, with respect to heparin, which can be found in the new derivatives, are in part also those which have already

been described for some heparin fragments obtained by alkaline treatment of heparin esters, for example desulfation and the presence of unsaturated monosic units.

The identification of the new derivatives can be carried out, apart from said chemical tests, by spectroscopy, especially nuclear magnetic resonance (NMR), which allows in particular to differentiate the new derivatives from heparin fragments already described in the literature.

The treatment of a quaternary ammonium salt of heparin with an alkylating agent chosen from those commonly used, that is, derived from a lower hydrocarbon, such as a lower alkyl halide, for example ethyl bromide, in a normally used solvent, such as dimethylsulfoxide or dimethylformamide, leads to heparin esters, such as those described in British patent No. 1501095. If these esters undergo alkaline treatment, such as that included in the second phase of the procedure of the present invention, different heparin fragments are obtained, as can be demonstrated by NMR analysis.

The new low-molecular-weight heparin derivative described in the examples, constitutes a novel product, which can be characterized mainly on the basis of NMR spectroscopic data at high frequency (400-500 MHz for the proton analysis; 100-125 MHz for C-13 analysis), utilizing traditional one-dimensional spectres in FT (Fourier Transformation), corroborated with the latest two-dimensional techniques (H-H and C-H correlation), optionally resorting to more recent methods (e.g. TOCSY = total correlation spectroscopy), where necessary for the study of such complex products.

From an overall examination of the results obtainable by the cited methods, it can be determined that:

1) The new "low-molecular-weight heparin" of the invention is a complex mixture of oligosaccharides, sulfated both at N and at O, reflecting the heterogeneity of the starting heparin. Its fundamental constituents IdoA and GlcnAc, differently sulfated, and secondary constituents (essentially GlcA) link together, giving rise to different sequential combinations.

2) With respect to the starting heparin, the product which is the object of the present invention shows a considerable decrease in the IdoA/GlcN ratio, this decrease being detectable by NMR. Accordingly, the signals of the anomeric sites of the typical IdoA sequence ($2SO_3$)-GlcNSO_3) ($6SO_3$) (102 and 99.5 ppm for carbon; 5.18 and 5.32 ppm for the proton) prove to be drastically diminished. Such transformations cannot be detected in the known, low-molecular-weight heparins, or if they can, it is to a far lesser degree. Concomitantly, markedly intense signals appear of a new unsaturated monosaccharide unit (see point 4).

3) Another characteristic fact is the appearance of new signals (at 54.2 and 53.3 ppm for carbon, correlatable to protons at 3.73 and 3.68 ppm respectively) which is compatible, in the present context, only with the 2-position of a GlcN residue, with no SO_3H groups at the nitrogen). This characteristic, detectable by 2-D NMR, is practically absent in samples of known, low-molecular-weight heparins.

4) Typically, most of the aforesaid oligosaccharides terminate with an unsaturated monosaccharide unit, three-substituted in positions 1-2, as can be shown by NMR (signals at 147 and 172 ppm in C-13: doublet at 6 ppm for the proton). These signals are absent or very reduced in the other known, low-molecular-weight heparins.

It must therefore be concluded that as early as the first step of the procedure of the present invention, what happens is not just a simple esterification, but a more complex chemical reaction.

It can therefore be assumed from these findings that the heparin-like products of the present invention are new and different from the heparin fragments already described in the literature. For example, they are different from the fragments described in European patent No. 0 302 034, obtained by alkaline treatment of heparin esters, these fragments being suitable for the formation of complexes with copper ions having an angiogenic action. In the same way, the heparin fragments described in U.S. patent No. 4,440,926, obtained starting from well-defined heparin esters by treatment with NaOH (0.1-0.5 N) between 20° and 60°C, are not identical to the products of the present invention.

The aforesaid qualities of the new heparin derivatives of the present invention can be demonstrated by the following experiments, performed on the product described in the illustrative Examples and abridged to the code name PE. In the experiments, unfractionated heparin (UFH) and a low-molecular-weight heparin derivative (CY 216 = Fraxiparine®) are used as comparison products. Both these products are commercially available for the treatment of thrombosis.

Brief Description of the Drawings

Figure 1 shows the anticoagulant activity in vitro of PE compared to UFH and CY 216 in terms of thrombin time (sec.). The data obtained are mean values of 7-10 replications for each test product.

Figure 2 shows the ex vivo anticoagulant activity of PE, compared to UFH and CY 216, according to the effect on thrombin time after i.v. administration. The data are mean values from experiments on 5 rabbits for each test product.

Figure 3 illustrates the ex vivo anticongulant activity of PE, compared to UFH and CY 216, based on anti-FXa activity (disappearance kinetics after s.c. administration). The data are mean values from experiments on 5 rabbits per test product.

Figure 4 shows the ex vivo activity for PF_4 after i.v. administration of PE, in comparison to UFH and CY 216.

1. ANTICOAGULANT ACTIVITY IN VITRO ON RECONSTRUCTED HUMAN PLASMA

Materials and Methods

Test products (solubilization and concentrations)
The following products were tested:
- heparin derivative PE
- unfractionated heparin (UFH)
- low-molecular-weight heparin derivative (CY 216 - Fraxiparine®)

The test products were dissolved in sterile saline and tested at concentrations ranging from 0.5 to 12.5 µg/ml.

Parameters

1. Thrombin time (as an indicator of anticoagulant activity)
2. Anti-FXa activity (as an indicator of anticoagulant activity)

Results

1. Thrombin time

From Fig. 1 it is clear that:

- PE has anticoagulant activity starting at concentrations of 1.25 µg/ml
- The anticoagulant activity of PE is considerably inferior to that of unfractionated heparin UFH (thrombin time increases at concentrations about 4-5 times greater than UFH).
  No significant differences from CY 216 can be observed.

2. Anti-FXa activity

In Table 1, the preliminary data indicate that:

- PE has anti-FXa activity starting from concentrations of 3 µg/ml
- the anti-FXa activity is distinctly inferior to that of heparin.
  It must also be noted that this activity is also inferior to that of CY 216; in this case, however, the difference is less evident than that observed in comparison to UFH.

Table 1

ANTI-FXa ACTIVITY IN VITRO OF PE IN COMPARISON TO UNFRACTIONATED HEPARIN AND CY 216

CONCENTRATIONS (μg/ml)	UFH	CY 216	PE
0.5	0.336	-	-
1	-	0.331	0.342
1.5	0.281	-	-
2.5	0.212	-	-
3	-	0.261	0.296
3.5	0.179	-	-
5	-	0.200	0.245
7	-	0.158	0.201

The data are means of 3 experiments per concentration, one for each test product.

2. ANTICOAGULANT ACTIVITY EX VIVO ON RABBIT PLASMA AFTER I.V. AND S.C. ADMINISTRATION

The anticoagulant activity of the new heparin derivative PE was assessed by ex vivo methods on rabbit plasma. By "ex vivo" is meant in vitro measurement of blood withdrawn from an animal to which the chemical substance has been administered. Examined in particular were the following:

a) thrombin time, on arterial blood after acute bolus intravenous administration (i.v.) of the test products;

b) anti-FXa activity, on arterial blood after acute

subcutaneous administration (s.c.).
This method, being highly sensitive, is indicative of the "bioavailability" of the test products, since it allows the kinetics of the disappearance of the test products to be monitored after subcutaneous administration.

The test products were dissolved in sterile saline solution and administered acutely at 0.86 mg/kg by the i.v. route and at 1-2 mg/kg by the s.c. route.

Test description

a) Thrombin time

This test assesses thrombin time on samples of arterial blood from rabbits at various time intervals (2-5-10-20-30-40 minutes) after i.v. administration of a bolus of test product.

b) Anti-FXa activity

By measuring the anti-FXa activity (by a chromogenic test which is sensitive at very low concentrations of compound) it is possible to monitor the presence of the test products in the circulation for a very long time. Samples of arterial blood (from the central artery of the ear) were taken at various intervals (1-2-3-4-5-6-7-8 hrs) after s.c. administration.

Results

a) Thrombin time

From Fig. 2 it is clear that:

- PE has a low anticoagulant activity at 0.86 mg/kg i.v., confirming the in vitro data
- the anticoagulant activity of PE is distinctly inferior to that of UFH, but does not differ significantly from that of CY 216.

b) anti-FXa activity

Fig. 3 shows that:

- PE (after s.c. administration at 1 mg/kg) remains present in the circulation for longer than UFH, administered at twice the dose, that is, at 2 mg/kg s.c.
  The disappearance kinetics of the heparin derivative PE is therefore slower than that of UFH, but similar to that of CY 216.

3. EX VIVO AFFINITY FOR PLATELET FACTOR 4 (PF_4)

By the experiments described hereafter, the affinity of the new heparin derivative PE of the present invention was assessed for human PF_4, i.e. the "binding properties" of such products for PF_4.

The ex vivo technique used monitored the kinetics of the disappearance of PF_4 from the circulation in the absence and in the presence of heparin.

It is well known that heparin or glycosaminoglycans (GAGs), administered before PF_4, markedly enhance its kinetics (G. Cella et al., "Human platelet factor 4 and protamine sulphate interaction with glycosaminoglycans in the rabbit", Eur. J. Clin. Invest., Vol. 17, pp. 548-554 (1987).

Compounds able to bind PF_4 should therefore greatly prolong its presence in the circulation. This effect is proportional to the quantity of GAG injected and to its affinity for PF_4.

The test products were dissolved in sterile saline solution and administered at 0.86 mg/kg i.v.

Description of the test

Administration of the test products was acute (intravenous bolus), at a dose of 0.86 mg/kg. Samples of arterial blood were taken 2-3 mins later from the central artery of the ear (baseline sample). 5 minutes after administration of the first bolus, a second bolus of purified human PF_4 was given (30 μg/kg).

Samples were taken 1.5-2.5-5-10-20-30 minutes after administration of PF_4. These were centrifuged, stored and assessed by the RIA test (commercially available kit) for human PF_4.

Results

As reported in Fig. 4, the data obtained indicate that:

- PE has affinity for platelet factor PF_4 and gradually disappears from the circulation reaching low values about 30 minutes after administration.
- The affinity of PE for PF_4 is much lower (about 60%) than that of UFH and the kinetics of the disappearance of the complex with PF_4 is far more rapid than that of UFH, while it is similar to that of CY 216.

4. ANTITHROMBOTIC ACTIVITY IN VIVO IN A MODEL OF ARTERIAL THROMBOSIS IN RABBIT

The experiment described hereafter assessed the antithrombotic activity in vivo of the new heparin derivative PE. The objective was to test the efficacy of the products in preventing the formation of an arterial thrombus in an acute model of arterial thrombosis in the rabbit carotid artery, following endothelial damage and reduction of the vascular

diameter.

The test products were dissolved in sterile saline and administered by the i.v. route at concentrations ranging from 1.2 to 6 mg/kg.

Description of the model

The experiments were performed on rabbits anesthetized with Nembutal (30 mg/kg i.v., bolus) and tracheotomized for forced breathing. A tube was inserted into the femoral vein and artery for the continuous administration of anesthetic and drug. Systemic blood pressure was monitored and the carotid was fixed with a flowmeter and snare. When the system stabilized, stenosis was performed. After suitable intervals (not less than 20 minutes), mechanical damage was induced with surgical forceps and stenosis was performed above the damaged vessel. The decrease in flow was monitored until it reached the value zero (0), corresponding to occlusion of the vessel. The controlateral carotid was prepared in the same way. The test products were then infused i.v. for eight minutes in all, with mechanical damage being performed after two minutes of infusion. The flow was monitored constantly until at least one hour after the end of treatment. The animal was sacrificed by an overdose of anesthetic at the end of the experiment.

Results

As reported in Table 2, the data obtained indicate that:

- PE is effective in completely preventing the formation of an occlusive thrombus. The effect is evident at a dose of 6 mg/kg i.v.;
- The antithrombotic efficacy of PE is inferior

(about 5x) to that of unfractionated heparin UFH (which can be seen to inhibit the formation of occlusive thrombi at a dose as low as 1.2 mg/kg); the pharmacological efficacy of PE is similar to that of CY 216.

Table 2

Antithrombotic activity of PE in comparison to unfractionated heparin (UFH) and CY 216 in a model of arterial stenosis in rabbit

Products	i.v. dose	No. occluded animals / No. treated animals
UFH	1.2 mg/kg	2/12
PE	6 mg/kg	2/12
	3 mg/kg	6/8
CY 216	6 mg/kg	2/12
	3 mg/kg	5/9

5. ANTITHROMBOTIC ACTIVITY IN A MODEL OF VENOUS THROMBOSIS IN VIVO

The experiment described hereafter was performed to assess the antithrombotic efficacy in vivo of PE in a model of venous thrombosis in the rat.

The venous stasis technique was used, by occluding the vena cava inferior, as this method causes the formation of a mainly fibrinic thrombus due to the variation in blood flow at the level of the bifurcation of the vena cava with the left renal vein.

The model under examination therefore proves to be sensitive to the activity of anticoagulant drugs and in particular to the antithrombotic potential of heparin (after administration and stasis).

The test products were dissolved in sterile saline and administered intravenously at doses ranging from 0.5 to 3 mg/kg i.v. (15 minutes before stasis induction).

Description of the test

The experiments were performed on male CD-COBS rats, weighing 175-200 grams. The animals were anesthetised with sodium pentobarbital (40 mg/kg i.p.) and their undersides were disinfected with alcohol. A 4-cm incision was made along the middle of the abdomen starting from the ribs. The vena cava was isolated from the aorta to the common iliac artery and tied with a cotton thread immediately underneath the fork with the left renal vein. The double occlusion was such as to exert constant and continuous pressure for 5 seconds. The abdominal wall was then closed. Two hours later the abdomen was reopened to verify thrombus formation. After closing off the vena cava, in correspondence to the common iliac arteries, and the collateral circulation with a hemostat, the vascular section below the occlusion was cut longitudinally with a needle. The thrombus was removed and immersed in distilled water, blotted dry on paper and then placed in a dry box for 24 hours to determine the dry residue expressed in mg.

Results

The data relative to the weight (mg) of the thrombi (Table 3) and incidence (%) of the thrombi (Table 4) two hours after venous stasis, show that:

- PE has antithrombotic efficacy; its protective effect (pre-treatment) is significant at a dose of 1.5 mg/kg i.v.
- the efficacy of PE is inferior to that of unfractionated heparin UFH (which already has a significant effect at 0.5 mg/kg i.v.)

Table 3

Antithrombotic effect in vivo of PE compared to UFH in a venous stasis model: weight (mg) of the thrombi obtained 2 hrs after venous stasis (effected 15 minutes after i.v. administration of the test products)

	Doses (mg/kg i.v.)					
Products	0 n = 15	0.5 n = 14	1.0 n = 24	1.8 n = 20	2.0 n = 14	3.0 n = 14
PE	2.2	1.4	1.5	0.8*	0.0**	0.0**
UFH	2.2	0.7*	0.5**	0.02**	-	-

* $p < 0.05$; ** $p < 0.01$ Dunnett's test

n = number of animals

Table 4

Antithrombotic effect in vivo of PE compared to UFH in a venous stasis model: incidence (%) of thrombi obtained 2 hours after venous stasis (effected 15 minutes after i.v. administration of the test products)

	Doses (mg/kg i.v.)					
Products	0 n = 15	0.5 n = 14	1.0 n = 24	1.5 n = 20	2.0 n = 14	3.0 n = 14
PE	93.3	85.7	62.5	60.0	0.0	0.0
UFH	93.3	57.1	50.0	14.3	-	-

n = number of animals

6. EFFECT ON BLEEDING TIME IN RAT

The experiment described hereafter assessed the effect of PE on bleeding time 15 minutes after intravenous administration of the test products.The test products were dissolved in sterile saline and administered i.v. at concentrations ranging from 1 to 3 mg/kg.

Description of the test

The experiments were performed on male, CD-COBS rats (175-200 grams) in which the test substances were administered acutely by the intravenous route.The bleeding time (in seconds) was assessed 15 minutes later by the standard template method of cutting the tail (Dejana et al., "Bleeding time in rats: A comparison of different experimental conditions", Thromb. Haemostat., Vol. 48, pp. 108-111 (1982)).

Results

The data obtained (Table 5) show that:

- The effect of PE on "bleeding time" is significantly increased at a dose of 2.0 mg/kg i.v.
- the effect of PE is notably inferior to that obtained with unfractionated heparin UFH (in which the bleeding value already increases significantly at a dose of 1 mg/kg i.v.), but it is comparable to that of CY 216 (data not reported).

It is interesting to note that in order to obtain the same values for the bleeding time it is necessary to administer a dose of PE which is at least 3 times greater than that of UFH.

Table 5

The effects of PE and UFH on bleeding time (sec) 15 minutes after i.v. administration of the test products

	Doses (mg/kg e.v.)				
Products	0 n = 14	1.0 n = 14	1.5 n = 14	2.0 n = 14	3.0 n = 14
PE	130	146	147	250*	389**
UFH	130	413**	487**	-	-

* $p < 0.05$; ** $p < 0.01$ Dunnett's test

n = number of animals

7. IN VIVO TOLERANCE AFTER I.V. AND S.C. ADMINISTRATION IN MOUSE

The present experiment assessed the maximum tolerated dose of the compound PE after administration by the intravenous and subcutaneous route in the mouse.

The test products were dissolved in sterile saline and tested at concentrations ranging between 125 and 2000 mg/kg i.v. and s.c.

Description of the test

The experiment was performed on male and female CD-1 mice (C. River) (25-35 grams) and comprised two steps.

1) Screening. Groups of one male mouse and one female mouse were treated with one of the following doses: 125, 250, 500, 1000 and 2000 mg/kg. Mortality within 7 days of treatment was monitored.
2) Groups of five male and five female mice were treated (for each compound and each administration route) with the highest tolerated dose found on screening. The animals were observed for 14 days, during which mortality and the presence of altered behaviour and general toxicity symptoms were observed. In the case of mortality a lower dose was used, until the maximum tolerated dose was found. Where possible, autopsy was performed on the deceased animals.

Results

1. Intravenous tolerance dose

As reported in Tables 6 and 7, the maximum tolerated dose of the test compounds proved to be the following:

- PE: 1000 mg/kg i.v.
- unfractionated heparin UFH: 125 mg/kg i.v.
- CY 216: 1000 mg/kg i.v.

It must be noted therefore that PE has greater tolerance dose than unfractionated heparin UFH.

Autopsy of the animals which died after treatment with unfractionated heparin UFH at 500 and 250 mg/kg, revealed no macroscopic findings except, in some animals, the presence of probable residues of internal hemorrhage in the intestine.

2. Subcutaneous tolerance dose

The results obtained, as reported in Tables 8 and 9, show that the maximum tolerated doses of the test products are as follows:

- PE: 2000 mg/kg s.c.
- unfractionated heparin UFH: 125 mg/kg s.c.
- CY 216: 2000 mg/kg s.c.

As with the intravenous route, therefore, the tolerance dose of PE is far greater than that of unfractionated heparin (UFH), while it is similar to that of CY 216. It is however important to highlight that:

- In all the animals (10/10) treated with CY 216 (2000 mg/kg s.c.) a circular area of skin affected by necrosis was observed on the back, corresponding to the injection site, the size of which differed from animal to animal (maximum diameter about 1 cm).

Autopsy revealed necrosis to be limited to the skin; it did not therefore affect the underlying muscle.

- No anomalies were observed in animals treated with PE (2000 mg/kg s.c.).
- The animals which died after treatment with UFH (250 mg/kg) presented extensive bruising in the subcutaneous dorsal region, where treatment had been performed.

The subcutaneous tolerance data therefore show a difference between the compounds PE and CY 216. Indeed, although the maximum tolerance doses are the same, in the case of CY 216 a marked area of necrosis of the skin is present at the injection site, which indicates that CY 216 is tolerated to a lesser degree locally than PE (which presented no anomalies).

Table 6

Lethality of compounds PE, CY 216 and unfractionated heparin UFH after a single intravenous administration (screening)

Treatment (mg/kg i.v.)	Deceased animals/Treated animals males	females
PE 2000	0/1	0/1
PE 1000	0/1	0/1
PE 500	0/1	0/1
PE 250	0/1	0/1
PE 125	0/1	0/1
CY216 2000	1/1	0/1
CY216 1000	0/1	0/1
CY216 500	0/1	0/1
Heparin UFH 2000	1/1	1/1
Heparin UFH 1000	1/1	1/1
Heparin UFH 500	0/1	0/1
Heparin UFH 250	0/1	0/1
Heparin UFH 125	0/1	0/1

Table 7

Lethality of compounds PE, CY 216 and unfractionated heparin (UFH) after a single intravenous administration

Treatment (mg/kg i.v.)	Deceased animals/Treated animals		Time of death				
	males	females		days after treatment			
			immediate*	1	2	3	4
PE 2000	3/7	1/5	2	-	1	-	1
PE 1000	0/5	0/5	-	-	-	-	-
CY216 1000	0/5	0/5	-	-	-	-	-
Heparin 500	0/5	2/5	-	2	-	-	-
UFH 250	3/5	0/5	-	3	-	-	-
125	0/5	0/5	-	-	-	-	-

*Within minutes of treatment

Table 8

Lethality of the compounds PE, CY 216 and unfractionated heparin UFH after a single subcutaneous administration (screening)

Treatment (mg/kg s.c.)	Deceased animals/Treated animals males	females
PE 2000	0/1	0/1
PE 1000	0/1	0/1
PE 500	0/1	0/1
PE 250	0/1	0/1
PE 125	0/1	0/1
CY216 2000	0/1	0/1
CY216 1000	0/1	0/1
CY216 500	0/1	0/1
Heparin UFH 2000	1/1	1/1
Heparin UFH 1000	1/1	0/1
Heparin UFH 500	1/1	0/1
Heparin UFH 250	0/1	0/1
Heparin UFH 125	0/1	0/1

Table 9

Lethality of compounds PE, CY 216 and unfractionated heparin (UFH) after a single subcutaneous administration

Treatment (mg/kg i.v.)	Deceased animals/Treated animals males	females	Time of death days after treatment				
			immediate	1	2	3	4
PE 2000	0/5	0/5	-	-	-	-	-
CY216 2000	0/5	0/5	-	-	-	-	-
Heparin 250	1/5	2/5	-	2	1	-	-
UFH 125	0/5	0/5	-	-	-	-	-

CONCLUSIONS

The in vitro and in vivo results previously described outline an interesting pharmacological profile of the heparin derivative PE, setting it apart from unfractionated heparin (UHF) and rendering its pharmacological activities more valuable.

The activity of PE differs slightly from that of CY 216, which is one of the best low-molecular-weight heparins currently on sale in France, and shows in particular better local tolerability after s.c. administration in the mouse. Indeed, although the maximum tolerated doses are the same, all animals treated with CY 216 (2000 mg/kg s.c.) had evident necrosis of the skin at the injection site, while the skin of the animals treated with PE was unaffected.

In the case of pharmacological activity in vitro and in vivo, it is interesting to note that PE has:

- anticoagulant activity, far inferior to that of UFH;
- bioavailability, after s.c. administration, much greater than that of UFH, thereby allowing fewer daily administrations for the same pharmacological effect;
- lesser side effects, with regard to UFH, as shown by the reduced bleeding time and by the absence of skin necrosis at the injection site, with regard to CY 216 (the best low-molecular-weight heparin on sale in France) which caused a local intolerance phenomena (necrosis) in all the animals treated ;
- antithrombotic activity in vivo, as shown in both the model of venous stasis in the rat and in the model of arterial thrombosis in the rabbit. The product is therefore efficacious in preventing these

pathologies at dosages of 1.5 and 6 mg/kg i.v., respectively, which do not induce any significant variations in the side effects.
In particular, with regard to the venous antithrombotic activity, the derivative PE, at a dose of 1.5 mg/kg i.v., does not induce any alterations in bleeding time, unlike UFH.

The new, low-molecular-weight heparin derivatives according to the present invention can therefore be used in the place of unfractionated heparin in all its indications, as an anticoagulant and thrombocyte aggregation inhibitor. The dose is adapted to each particular case, and is normally around 1-7 mg/kg per day, by the intravenous route.

The heparins to be used in the previously described procedure of the present invention can be of any type and of various origin, for example heparins extracted from the intestine of pigs, cattle, and sheep and from ox heart, especially any one of the products which are commercially available or described in the literature. Such products have widely ranging molecular weights, for example between 2,000 and 30,000 daltons, especially unfractionated heparin UFH, and also low-molecular-weight heparins obtained by fractionation of standard heparins (2,000-10,000 daltons) and heparin fragments with a molecular weight of 500-10,000 daltons obtained by partial depolymerization of standard heparins by chemical or enzymatic means. The quaternary ammonium salts to be used as starting material for said procedure can be prepared in the known way, for instance by ion exchange of the heparin in aqueous solution as a sodium or potassium salt with resins based on quaternary ammonium salts, for example a salified sulfonic resin with a

quaternary ammonium base. The quaternary ammonium salt can be obtained by freeze-drying the eluate. As quaternary ammonium salts, tetraalkylammonium salts derived from lower alkyls may be advantageously used, particularly tetraalkylammonium salts with alkyl groups having a maximum of 6 carbon atoms. Optionally, alkyl-aryl-ammonium salts may also be used, for example those having long-chained alkyl groups. Of the tetraalkylammonium salts, tetrabutylammonium salts are preferably used. The quaternary ammonium salts to be used as starting substance for the procedure of the present invention are those obtainable by ion exchange in the aforesaid manner using alkaline salts, for example sodium or potassium salts, of heparin of the type used commercially, that is, neutral salts. They are reacted with an excess of quaternary ammonium ions so as to obtain also the corresponding neutral salts.

Quaternary ammonium salts are soluble both in the aforesaid heterocyclic solvents, and in aprotic organic solvents, such as dimethylsulfoxide and dimethylformamide. The reaction with the alkylating agent, according to the first step of the procedure, can be performed both in the aforesaid heterocyclic solvents, or in a solution of the same in one of the aforesaid aprotic organic solvents, preferably in a concentrated solution. Of the aforesaid heterocyclic solvents, the preferred ones are above all those which are not substituted in the cyclic structure, that is, N-alkyl-o-aryl-2-pyrrolidone, or their derivatives containing a heterocyclic atom in the cyclic structure, such as the corresponding derivatives of imidazoline, piperazine or morpholine. The N-alkyl groups of heterocyclic solvents are derived preferably from a lower alkyl with a maximum of 6 carbon atoms, and the aryl

group is primarily a phenyl group, optionally substituted by 1 to 3 lower alkyl groups, especially methyl groups. Preferably, N-methyl-2-pyrrolidone is used.

An alkylating agent derived from a hydrocarbon having from 6 to 30 carbon atoms, preferably from 8 to 18 carbon atoms, is a compound easily capable of yielding the corresponding hydrocarbyl groups, such as a reagent ester group of an acid with an alcohol with a corresponding number of carbon atoms, which can be an aliphatic or araliphatic alcohol having preferably a maximum of 18 carbon atoms, for example a hexyl, heptyl, octyl or nonyl alcohol. The reagent esters can be derived from inorganic or organic acids, such as hydracids, sulfuric or sulfurous acid, or alkyl- or aryl-sulfonic acids, for example methane-sulfonic or p-toluenesulfonic acid. The esters of the hydracids are in particular chlorides, bromides and iodides. The reaction between the quaternary ammonium salt of the aforesaid alkylating agent is performed at room temperature (20°C.)or slightly higher, for example at a temperature of from 30 - 35°C. but not exceeding 60°C, and is maintained for a prolonged period of time of about 5 to 20 hours, typically about 16 hours.

The reaction product can be isolated and subsequently submitted to the second step of the procedure, that is, to alkaline treatment, or it can be converted directly into the final product, performing the alkaline treatment directly on the solution, optionally concentrated, of the reaction product of the first step. It is also possible to evaporate the solvent under bland (mild) conditions after the first step of the procedure and to perform alkaline treatment on the residue. It is therefore possible to perform the procedure of the invention as a "one-pot" process. The basic treatment of

the reaction product between the quaternary ammonium salt of heparin with the alkylating agent is performed at a temperature of from 5° to 120°C., suitably at a temperature between 50°C and 120°C, preferably about 70°C, for about two hours. Alkaline hydrates are used, such as NaOH or KOH, or other inorganic and/or organic bases, preferably at a concentration of between 0.1 and 1M. The bases are used in aqueous or alcoholic solutions, but other solvents can be present which are miscible with water or with an aqueous-alcoholic mixture, such as, for example, the residue solvents used in the first step of the procedure in the case of the aforesaid "one-pot" process.

If it is desired to isolate the reaction product of the first step of the procedure, it can be precipitated by the addition of an organic polar solvent, preferably an aliphatic alcohol such as methyl or ethyl alcohol, to which a basic buffer has preferably been added, such as especially a basic salt of a carboxylic acid, e.g., an acetate or propionate of sodium or potassium. Other basic buffers can be used, such as alkaline bicarbonates or alkaline phosphates. The precipitated product is preferably washed with the polar solvent used in the precipitation, for example methanol. It is advisable to purify the precipitated product further by reprecipitating it once more or several times, that is, by dissolving the product in water and precipitating it with an alcohol.

The reaction product, comprising the low-molecular-weight heparin derivative and having the previously described properties, can be released in free form either from a metal salt or from an organic base in the conventional way. The alkaline salt corresponding to the alkaline hydrate used in the second step of the

procedure may be obtained by neutralizing the solution with an acid, e.g., 2M hydrochloric acid, purifying the solution, extracting it with an organic solvent which cannot be mixed with water, for example methylene chloride, and repeating this operation several times. The salt is then dialyzed with distilled water and sodium chloride and freeze-dried.

The products of the invention are used mainly in the form of their metal salts or organic base salts. If it is desired to obtain metal salts other than alkaline salts corresponding to the ions of the alkaline hydrates used in the aforesaid alkaline treatment, it is possible to use ion exchange methods by known techniques. Alternatively, it is possible to isolate the heparin product in its acid form, adding the alkaline salt obtained with the calculated quantity of a diluted strong acid, such as hydrochloric acid or sulfuric acid, extracting the product with a suitable organic solvent, isolating the heparin compound in free form and then converting it into the desired salt.

Of the metal or organic base salts which can be used according to the present invention, of particular importance are those which are therapeutically acceptable, such as alkali and alkaline-earth metals, for example sodium, potassium, ammonium, calcium, and magnesium salts, or heavy metal salts, such as copper and iron salts. The salts of organic bases can be derived from primary, secondary or tertiary aliphatic, aromatic or heterocyclic amines, such as methylamine, ethylamine, propylamine, piperidine, morpholine, ephedrine, furfurylaminecholine, ethylenediamine and aminoethanol.

Salts which cannot be directly used in therapy, which are also part of the invention, are for example those which can be used for the purification of the new

heparin derivatives of the invention, such as some of the heavy metal salts.

The invention also includes pharmaceutical preparations containing as active substance a new low-molecular-weight heparin derivative obtainable by the procedure described herein, especially in the form of their therapeutically acceptable salts. Such preparations can be for parenteral use, for example for subcutaneous or intravenous administration, or for topical use, for example in the form of creams or ointments, or suppositories, or nose sprays. They can therefore be formulated as solutions of the active compounds or as freeze-dried powders of the active compounds to be mixed with one or more pharmaceutically acceptable excipients or diluents which are convenient for the aforesaid uses and with an osmolarity which is compatible with physiological fluids.The invention includes, besides the new low-molecular-weight heparin derivatives, the aforesaid procedure for their preparation. Included in the invention is also the performance of the single steps of the preparation procedure, that is, the reaction of a quaternary ammonium salt of heparin with an alkylating agent as defined above in one of said solvents and the alkaline treatment of the reaction product.

The invention is illustrated in the following Examples which, however, are not to be considered as limiting.

1) FORMULATIONS FOR INJECTION

1.1

PE	mg	10	20	30	40
water for injection in	ml	0.1	0.2	0.3	0.4

Package with ready-to-use syringe containing sterile solution for subcutaneous administration.

1.2

PE	mg	150	300
other components:			
water for injection in	ml	4	8

Package with vial containing sterile solution for intravenous administration.

1.3

PE	mg	10	20	30	40
other components:					
sodium chloride	mg	0.3	0.6	0.9	1.2
water for injection in	ml	0.1	0.2	0.3	0.4

Package with ready-to-use syringe containing sterile solution for subcutaneous administration.

1.4

PE	mg	150	300
other components:			
sodium chloride	mg	35	70
water for injection in	ml	5	10

Package with vial containing sterile solution for intravenous administration.

1.5

PE	mg	150	300
other components:			
sodium chloride	mg	35	70
sodium metabisulfite	mg	5	10
chlorbutanol (*)	mg	25	50
water for injection in	ml	5	10

Package with vial containing sterile solution for multidose intravenous administration.

(*) in Example 1.5 the preservative chlorbutanol can be substituted by:

benzyl alcohol	mg	50	100

or by:

chlorocresol	mg	5	10

2) FORMULATIONS FOR ORAL ADMINISTRATION

2.1 (pill or capsule)

PE	mg	30	60	120
other components:				
phosphatidylcholine + phosphatidylserine (*)	mg	80	160	320
lactose	mg	50	100	200
microcrystalline cellulose	mg	10	20	40
talc	mg	3	6	12
colloidal silica	mg	3	6	12

(*) the ratios between the phosphatidylcholine and phosphatidylserine present in the formulations may vary

2.2 (pill or capsule)

PE	mg	30	60	120
other components:				
phosphatidylcholine + phosphatidylserine (*)	mg	80	160	320
cholesterol	mg	2	4	8
lactose	mg	50	100	200
microcrystalline cellulose	mg	10	20	40
talc	mg	3	6	12
colloidal silica	mg	3	6	12

(*) the ratios between the phosphatidylcholine and phosphatidylserine present in the formulations may vary.

2.3 (packet of powder)

PE	mg	60	120	180
other components:				
phosphatidylcholine +				
phosphatidylserine (*)	mg	250	500	750
cholesterol	mg	5	10	15
lactose	mg	100	200	300
flavoring and				
sweetener as required				
sucrose (**) to	mg	2000	4000	6000

(*) the ratios between the phosphatidylcholine and phosphatidylserine present in the formulations may vary

(**) sucrose may be substituted by fructose

2.4 (retard effect tablet)

PE	mg	30	60	120
other components:				
phosphatidylcholine +				
phosphatidylserine (*)	mg	80	160	320
lactose	mg	30	60	120
microcrystalline				
cellulose	mg	5	10	20
hydroxypropyl-				
methylcellulose	mg	10	20	40
talc	mg	3	6	12
colloidal silica	mg	3	6	12

(*) the ratios between the phosphatidylcholine and phosphatidylserine present in the formulations may vary

2.5 (gastroresistant tablet)

PE	mg	30	60	120
other components:				
phosphatidylcholine +				
phosphatidylserine (*)	mg	80	160	320
lactose	mg	50	100	200
microcrystalline				
cellulose	mg	10	20	40
talc	mg	30	60	120
colloidal silica	mg	3	6	12
methacrylic acid				
copolymer	mg	15	30	60

(*) the ratios between the phosphatidylcholine and phosphatidylserine present in the formulations may vary

2.6 (gastroresistant, retard effect tablet)

PE	mg	30	60	120
other components:				
phosphatidylcholine +				
phosphatidylserine (*)	mg	80	160	320
lactose	mg	50	100	200
microcrystalline				
cellulose	mg	10	20	40
hydroxypropyl-				
methylcellulose	mg	5	10	20
talc	mg	30	60	120
colloidal silica	mg	3	6	12
methacrylic acid				
copolymer	mg	15	30	60

(*) the ratios between the phosphatidylcholine and phosphatidylserine present in the formulations may vary

2.7 (solution to be administered orally as drops)

PE	mg	30	60	120
other components:				
citric acid	mg	7.5	15	30
purified water to	ml	0.25	0.5	1

2.8 (granules *)

PE	mg	30	60	120
other components:				
citric acid	mg	7.5	15	30
sucrose	mg	30	60	60
corn starch	mg	12.5	25	25
talc	mg	5	10	10
polyvinylpyrrolidone	mg	4	8	8

(*) 3 granules administered in a hard gelatin capsule

2.9 (gastroresistant granules *)

PE	mg	30	60	120
other components:				
citric acid	mg	7.5	15	30
sucrose	mg	30	60	60
corn starch	mg	12.5	25	25
talc	mg	5	10	10
polyvinylpyrrolidone	mg	4	8	8
methacrylic acid copolymer	mg	10	15	15

(*) 3 granules administered in a hard gelatin capsule

3. NASAL OR PULMONARY FORMULATIONS

3.1

PE fine white powder	mg	20	40

The active principle, as a fine white powder, is contained in a gelatin capsule to be broken open when ready for use and loaded into suitable apparatus for inhalation.

3.2 (powder for inhalation)

PE fine white powder	mg	20	40
phosphatidylcholine + phosphatidylserine *	mg	30	60

The active principle, as a fine white powder, is contained in a gelatin capsule to be broken open when ready for use and loaded into suitable apparatus for inhalation.

(*) the ratios between the phosphatidylcholine and phosphatidylserine present in the formulations may vary

4. FORMULATIONS FOR RECTAL ADMINISTRATION

4.1

PE	mg	30	120
semisynthetic glyceride	mg	1940	1880

4.2

PE	mg	30	120
phosphatidylserine + phosphatidylcholine	mg	100	200
semisynthetic glyceride	mg	1840	1680

In summary, the low molecular weight heparin derivatives and salts thereof according to the present invention are obtainable by treatment of a quaternary ammonium salt of a heparin, dissolved in a heterocyclic organic solvent chosen from the group formed by N-alkyl-pyrrolidine-2-one, N-alkyl-piperidine-2-one, unsubstituted or substituted by lower alkyl groups, and their derivatives interrupted in the heterocyclic ring by another heteroatom or heterogroup selected from -O-, -S-, and -NH-, or in a concentrated solution of said compound in an aprotic solvent, with an alkylating (etherifying) agent derived from a hydrocarbon having from 6 to 30 carbon atoms, preferably from 8 to 18 carbon atoms, at a temperature of from about 20° (room temperature) to 60°C. for a prolonged period of time, e.g., about 5 to 20 hours. The resulting reaction product is then treated at a temperature of from 5° to 120°C., preferably about 70°C., with an inorganic or organic base in an aqueous solution. The product resulting from this alkaline treatment is then isolated in free form or as an alkali metal or alkaline earth metal salt thereof. Salts of other metals or organic base salts may be obtained therefrom by conversion from one to the other. Preferably, in the second step of the procedure, the reaction product is treated with an alkaline hydroxide in aqueous solution. The resulting low molecular weight heparin derivatives and salts thereof are useful therapeutically, for example, as anti-thrombotic agents.

CLAIMS

1. A process for preparing low molecular weight heparin derivatives and/or salts thereof which is characterized by

(1) treating a quaternary ammonium salt of a heparin dissolved in a heterocyclic organic solvent selected from N-alkyl-pyrrolidine-2-one, N-alkyl-piperidine-2-one, unsubstituted or substituted by lower alkyl groups, or derivatives thereof interrupted in the heterocyclic ring by another heteroatom or heterogroup selected from -O-, -S- or -NH-, or a concentrated solution of said compound in an aprotic solvent, with an alkylating agent derived from a hydrocarbon having from 6 to 30 carbon atoms, at room temperature or slightly higher,

(2) treating the resulting reaction product, at a temperature of from 5° to 120°C., with an inorganic or organic base in an aqueous solution, and

(3) isolating the heparin derivatives obtained in their free form or as alkali metal or alkaline earth metal salts thereof.

2. A process according to claim 1, which further comprises converting said heparin derivatives or salts obtained into salts of other metals or into organic base salts.

3. A process according to claim 1, which further comprises converting the heparin derivatives obtained in free form into pharmaceutically acceptable salts or converting said salts into heparin derivatives in free form.

4. A process according to claim 1, wherein in step (2) said reaction product is treated with an alkaline hydroxide in aqueous solution.

5. A process according to claim 1 or claim 4, wherein the product resulting from the reaction of the alkylating agent with the quaternary ammonium salt of heparin is isolated before being treated with the inorganic or organic base.

6. A process according to claim 1 or claim 4, wherein quaternary ammonium salts of heparin having molecular weights varying from 2,000 to 30,000 daltons are used as a starting material.

7. A process according to claim 1 or claim 4, wherein quaternary ammonium salts of unfractionated heparin are used as a starting material.

8. A process according to claim 1 or claim 4, wherein quaternary ammonium salts of low molecular weight heparin, obtained by fractionation of heparin having a molecular weight of 2,000 to 10,000 daltons and heparin fragments having a molecular weight of 500 to 10,000 daltons obtained by partial chemical or enzymatic depolymerization of heparin, are used as a starting material.

9. A process according to claim 1 or claim 4, wherein said quaternary ammonium salt is a tetraalkylammonium salt wherein the alkyl groups have from 1 to 6 carbon atoms.

10. A process according to claim 1 or claim 4, wherein said quaternary ammonium salt is the tetrabutyl ammonium salt.

11. A process according to claim 1 or claim 4, wherein the N-alkyl group in said heterocyclic organic solvent is a lower alkyl having from 1 to 6 carbon atoms.

12. A process according to claim 1 or claim 4, wherein said heterocyclic organic solvent is N-methyl-pyrrolidone.

13. A process according to claim 1 or claim 4, wherein an ester of an acid with an aliphatic or araliphatic alcohol having from 6 to 30 carbon atoms is used as the alkylating agent.

14. A process according to claim 13, wherein said alkylating agent has from 8 to 18 carbon atoms.

15. A process according to claim 14, wherein said alkylating agent is an iodide, bromide or alkyl chloride, or an ester of an alkyl- or arylsulfonic acid.

16. A process according to claim 1 or claim 4, wherein the alkylating agent is reacted with the quaternary ammonium salt of heparin dissolved in said heterocyclic organic solvent at a temperature of from about 20° to 60°C.

17. A process according to claim 1 or claim 4, wherein the reaction product resulting from the reaction of the alkylating agent with the quaternary ammonium salt

of heparin is isolated by precipitation by the addition of a polar organic solvent.

18. A process according to claim 17, wherein said polar organic solvent is an aliphatic alcohol.

19. A process according to claim 18, wherein a basic buffer is added to said aliphatic alcohol.

20. A process according to claim 19, wherein said basic buffer is a basic salt of a carboxylic acid.

21. A process according to claim 17, wherein the precipitated product is reprecipitated several times with the same organic solvent.

22. A process according to claim 1, wherein in step (2) said reaction product is treated with an alkaline hydroxide or with another inorganic and/organic base at a concentration of between 0.1 and 1M in aqueous solution or alcoholic-aqueous solution at a temperature of from 5° to 120°C.

23. A process according to claim 22, wherein the alkaline treatment step is performed on the isolated reaction product resulting from step (1) with 0.1 - 1M aqueous sodium hydroxide at a temperature of about 70°C. for a sufficient time to obtain said low molecular weight heparin derivatives and/or salts thereof.

24. A process according to any of the preceding claims, wherein the reaction product obtained from step (2) is isolated by neutralization of the alkaline aqueous solution, extraction with a water-immiscible organic

solvent, and dialysis with distilled water and sodium chloride.

25. A process for preparing lower molecular weight heparin derivatives and/or salts thereof which is characterized by

(1) treating the tetrabutylammonium salt of heparin having a molecular weight of 15,000 daltons dissolved in N-methyl-pyrrolidone with 1-iodooctane at room temperature for about 16 hours,

(2) precipitating the reaction product by pouring the resulting solution into methanol to which sodium acetate has been added,

(3) purifying the resulting product several times by reprecipitation from methanol to which sodium acetate has been added,

(4) treating the dried product with 0.5 N aqueous sodium hydroxide for about two hours at approximately 70°C.,

(5) neutralizing the resulting solution with 2M hydrochloric acid,

(6) extracting the solution several times with methylene chloride,

(7) dialyzing the solution with distilled water and 0.1 M sodium chloride, and

(8) freeze-drying the obtained solution.

26. Low molecular weight heparin derivatives and salts thereof, prepared by the process as recited in any of claims 1 - 25.

27. Alkali metal or alkaline earth metal salts of low molecular weight heparin derivatives prepared by the process as recited in any of claims 1 - 25.

28. A sodium, potassium or calcium salt of the heparin derivatives prepared according to any of claims 1 - 25.

29. Salts of therapeutically acceptable bases of the heparin derivatives prepared according to any of claims 1 - 25.

30. Sodium, potassium or calcium salt of the heparin derivatives prepared according to claim 29.

31. A pharmaceutical composition comprising low molecular weight heparin derivatives according to claim 26 as an active ingredient and a pharmaceutically acceptable carrier, diluent or excipient.

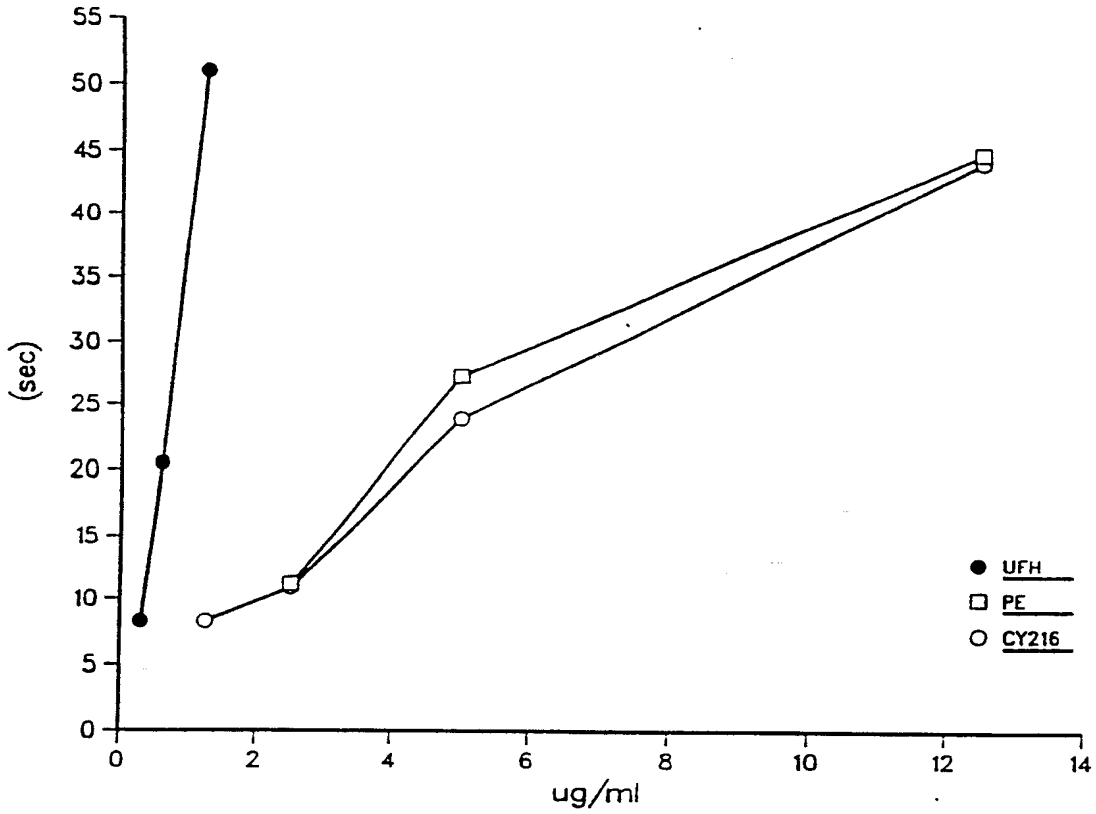
32. A pharmaceutical composition comprising an alkali metal or alkaline earth metal salt of low molecular weight heparin derivatives according to claim 26 as an active ingredient and a pharmaceutically acceptable carrier, diluent or excipient.

33. The use of low molecular weight heparin derivatives according to claim 26 as a therapeutic agent.

34. The use of alkali metal or alkaline earth metal salts of low molecular weight heparin derivatives according to claim 27 as a therapeutic agent.

35. The use of low molecular weight heparin derivatives or salts thereof according to claim 26 for the treatment of thrombosis.

36. A pharmaceutical composition according to claims 31 and 32 for use in the treatment of thrombosis.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50
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25
20
15
10
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(sec)
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2
4
6
8
10
12
14
ug/ml
UFH
PE
CY216

FIGURE 1

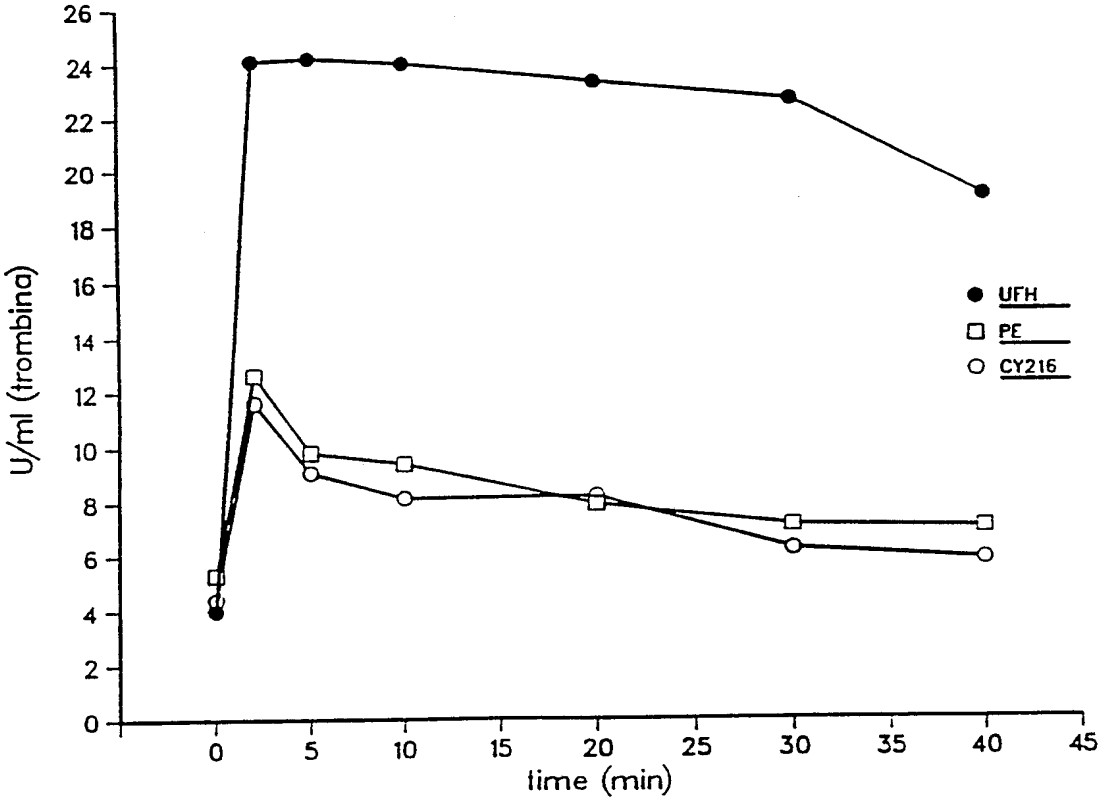

FIGURE 2

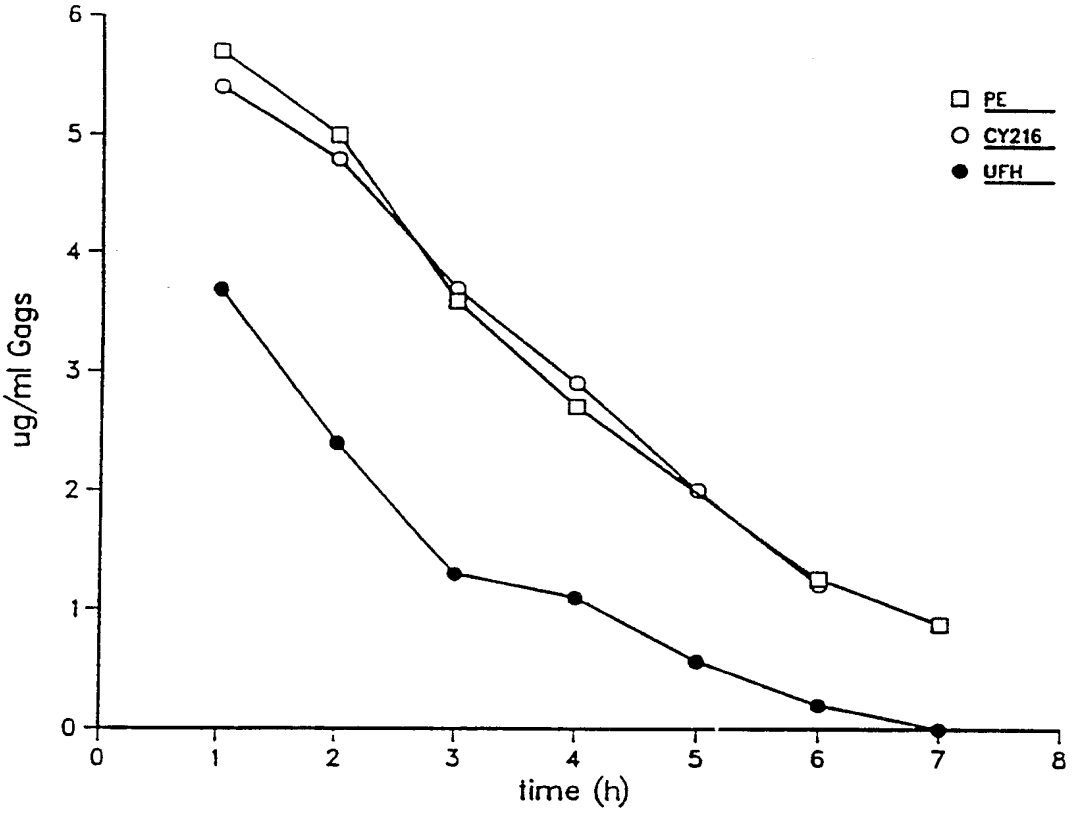

FIGURE 3

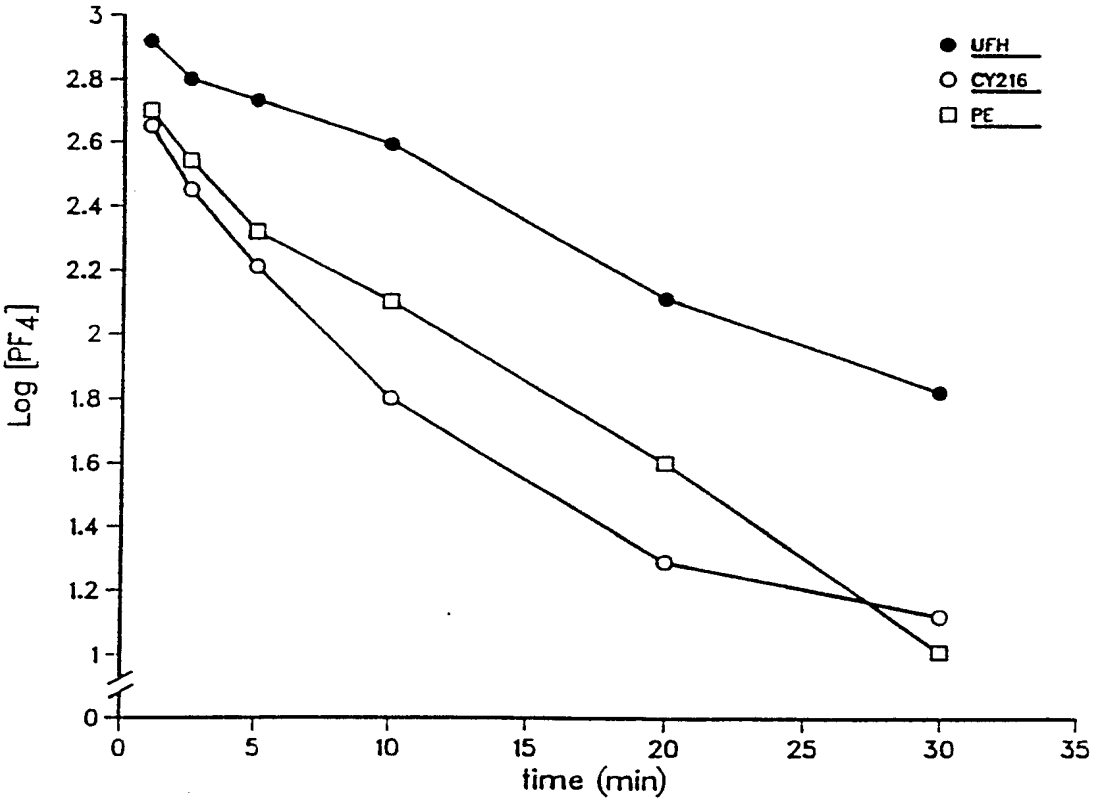
UFH
CY216
PE
Log [PF4]
time (min)
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
0
5
10
15
20
25
30
35

FIGURE 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 91/02479

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C08B37/10; A61K31/725

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C08B ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	EP,A,0 302 034 (KABIVITRUM AB) 1 February 1989 cited in the application see page 10; example 2 ---	1,4,5,7, 16-22
A	FR,A,2 482 603 (PHARMINDUSTRIE) 20 November 1981 see page 4, line 8 - page 6, line 5 & US,A,4 440 926 (J. MARDIGUIAN) 3 April 1984 cited in the application ---	33-35
A	EP,A,0 256 880 (MIAMI UNIVERSITY) 24 February 1988 see examples 1,7 ---	1

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28 FEBRUARY 1992	25. 03. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MAZET J.

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9102479 SA 54394

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 28/02/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0302034	01-02-89	AU-A- 1936988	21-12-88
		EP-A- 0319559	14-06-89
		JP-T- 1503548	30-11-89
		WO-A- 8809347	01-12-88
		US-A- 5039529	13-08-91
FR-A-2482603	20-11-81	AU-B- 543032	28-03-85
		AU-A- 7052081	19-11-81
		CA-A- 1183132	26-02-85
		EP-A,B 0044228	20-01-82
		JP-C- 1273970	11-07-85
		JP-A- 57008201	16-01-82
		JP-B- 59052163	18-12-84
		US-A- 4440926	03-04-84
US-A-4440926	03-04-84	FR-A- 2482603	20-11-81
		AU-B- 543032	28-03-85
		AU-A- 7052081	19-11-81
		CA-A- 1183132	26-02-85
		EP-A,B 0044228	20-01-82
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		JP-A- 57008201	16-01-82
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